Tetrahedron Vol. 49, No 1, pp. 13-28, 1993
Printed in Great Britain

0040-4020/93 $5.00+.00

A New Anti-MRSA Dipeptide, TAN-1057 A

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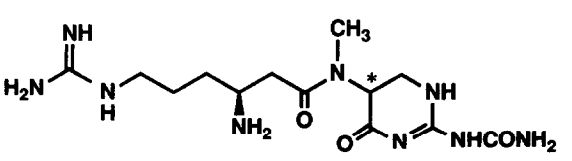
(*Received in Japan* 13 *July* 1992)

Abstract The chemical structure of a new dipeptide antibiotic, TAN-1057 A, isolated from the broth filtrate of *Flexibacter* sp. PK-74 was determined to be (3'*S*, 5*S*)-5-[*N*-methyl-*N*-(3'-amino-6'-guanidinohexanoyl)amino]-5,6-dihydro-2-ureido-4(1H)-pyrimidone. The antibiotic was specifically active against staphylococcus species including methicillin-resistant strains.

Recently, there have been an increasing number of cases of infections by Staphylococci, Streptococci, Enterococci and other bacteria resistant to β-lactams, macrolides and tetracyclines. In particular, methicillin-resistant *Staphylococcus aureus* (MRSA) infection is the cause of many clinical problems in hospitals.[1] We were thus stimulated to search for new antibiotics having novel action mechanisms against such microorganisms.

In our screening system, a bacterium from a soil sample of the Nachi mountain area in Wakayama Prefecture, Japan, *Flexibacter* sp. PK-74,[2] was found to produce the new peptides TAN-1057 A (**1a**) and B (**1b**). The antibiotics isolated from the broth filtrate were basic, water-soluble substances existing in an equilibrium with each other. Analyses of their physicochemical properties and spectral data showed the molecular formulas to be $C_{13}H_{25}N_9O_3$. They are dipeptides consisting of β-homoarginine and a pyrimidone derivative of 2,3-diaminopropionic acid as shown in Fig. 1. These antibiotics showed specifically strong therapeutic effects against infections of staphylococcus species including MRSA.

This paper deals with the isolation, chemical characterization and structure determination of TAN-1057.



*S, TAN-1057 A (**1a**)
*R, TAN-1057 B (**1b**)

Fig. 1 Structures of TAN-1057 A and B

The bioactive fractions were detected by antimicrobial activities against *S. aureus* in the early isolation stages and by analytical reversed-phase HPLC in the later isolation stages. Bioactive components in the broth filtrate were adsorbed on cation-exchange resins, such as Amberlite IRC-50, CG-50 or CM-Sephadex C-25. Desalination of eluates was carried out by chromatography on carbon or adsorptive resins, such as Diaion SP-207. The crude powder obtained was separated into two fractions by preparative reverse-phase HPLC using an ODS (octadesylsilane) column with a mobile phase of 0.1 M phosphate buffer (pH 5.0). Detailed HPLC analysis revealed TAN-1057 to be an equilibrium mixture of **1a** and **1b**. Treatment of **1a** in NaOMe-MeOH gave a 1:1 mixture of **1a** and **1b** ("**a**" and "**b**" next to the compound number indicate the configurations of the 2,3-diaminopropionic acid moieties to be the **1a**-type (*S*) and the **1b**-type (*R*) in this paper).

TAN-1057 A and B show positive color reactions to Dragendorff, Greig-Leaback and Sakaguchi. These antibiotics are easily soluble in dimethyl sulfoxide (DMSO) and water, moderately soluble in methanol, and insoluble in acetone, ethyl acetate or chloroform. The physicochemical properties of optically pure **1a** and **1b**, obtained *via* chemical modifications, are summarized in Table 1. The molecular formula of **1a** was determined from the elemental analysis, SIMS and ^{13}C NMR spectrum of its dihydrochloride. The UV spectrum indicated two maxima at 215 and 245 nm. The IR absorption pattern looked like that of a peptide compound. The ^{13}C NMR spectrum of **1a** indicated the presence of one *N*-methyl, two *N*-methylenes, three guanidino-like functions and two carbonyls in the molecule. The ^{13}C NMR spectra of the major derivatives are summarized in Table 2. H-H and C-H COSY revealed that **1a** had two moieties, $-CH_2-CH_2-CH_2-\underset{|}{C}H-CH_2-$ and $-CH_2-\underset{|}{C}H-$, as mentioned later. The CD spectra of **1a** and **1b** were symmetrical to each other and a 1:1 mixture of **1a** and **1b** showed no Cotton effect, as can be seen from Fig. 2. The specific rotations indicated opposite signs. These findings suggested that **1b** was an epimer of **1a**.

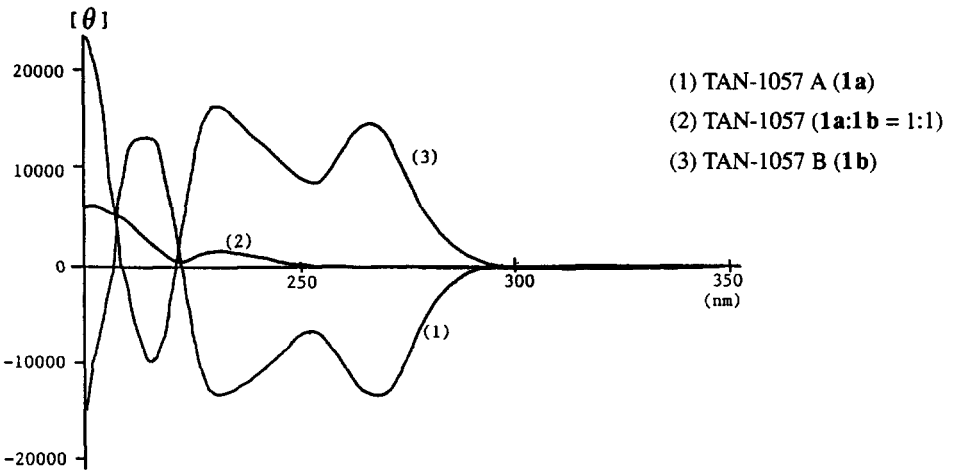


Fig. 2 CD Spectra of TAN-1057 ·2HCl

Table 1 Physicochemical Properties of TAN-1057 A and B (2 HCl)

Property		TAN-1057 A (**1a**)	TAN-1057 B (**1b**)
$[\alpha]_D^{22}$		-39.1°(c 0.530)	+72.6°(c 0.522)
SIMS *m/z*		356 $(M+H)^+$	356 $(M+H)^+$
Formula		$C_{13}H_{25}N_9O_3 \cdot 2$ HCl	$C_{13}H_{25}N_9O_3 \cdot 2$ HCl
UV λmax nm (ε)		215 (23,200)	215 (21,700)
		245 (11,800)	245 (11,200)
CD [θ] (nm)		+ 13,300 (215)	- 10,700 (215)
		- 13,500 (231)	+ 16,100 (230)
		- 13,500 (267)	+ 14,100 (266)
IR ν cm^{-1}		1720, 1610, 1570	1720, 1610, 1570
HPLC Rt (min)	1)	5.7	5.3
	2)	33.6	28.2

Column: ODS, YMC Pack A-312

Mobile phase: 1) 0.1 M phosphate buffer (pH 5.0); flow rate, 2.0 ml/min; 25℃

2) 0.01 N $HClO_4$ (pH 2.0); flow rate, 1.0 ml/min; 0℃

Table 2 ^{13}C NMR Chemical Shifts* of TAN-1057 Derivatives** (D_2O)

C No.	**1a**	**1b**	**2**	**3**	**4**	**5**	**15a**	**17a**	**18a**	**22b**
guanidino	159.7	159.7	159.7	-	-	-	159.7	159.7	159.6	159.7
6'	43.5	43.5	43.4	-	-	-	43.5	43.4	43.4	43.6
5'	27.0	27.0	26.9	-	-	-	27.0	26.9	26.9	27.4
4'	32.1	32.1	32.0	-	-	-	32.0	32.1	32.0	35.0
3'	51.2	51.2	51.0	-	-	-	51.3	51.3	51.1	52.7
2'	37.8	37.7	38.9	-	-	-	37.7	37.6	37.6	41.5
1'	175.3	175.3	177.2	-	-	-	175.4	175.3	175.3	173.0
2	160.6	160.6	-	160.3	156.6	-	157.0	159.9	159.2	157.2
4	178.5	178.5	-	172.5	167.5	172.0	177.1	177.4	174.2	176.6
5	56.4	56.6	-	63.4	55.9	60.3	61.3	62.6	57.0	65.3
6	41.4	41.5	-	42.7	40.1	39.9	37.8	43.2	40.8	45.2
N-Me	36.9	37.2	-	34.8	34.3	34.7	35.7	35.7	37.5	37.9
ureido	161.9	161.8	-	-	-	-	158.8	-	-	158.7

* δ values were recorded in ppm downfield from sodium 3-trimethylsilylpropionate-d_4

** 2 HCl salts except **17a** (2.5 HCl salt) and **18a** (3 HCl salt)

On acidic degradation of **1a** in 6 N HCl with refluxing for 18 hours, four amino acid analogs were isolated by chromatographies using cation exchange resins. One of them was (3*S*)-β-homoarginine[3](**2**) and three other products were derivatives of 2, 3-diaminopropionic acid as shown in Fig. 3. Compounds **3** and **4** existed as an 4:1 equilibrium mixture in aqueous solutions, but **4** was preferentially crystallized from methanol / diethyl ether. Degradation product **5** was determined to be 3-amino-2-methylaminopropionic acid[4,5] and regarded as a racemic compound from its specific rotation.

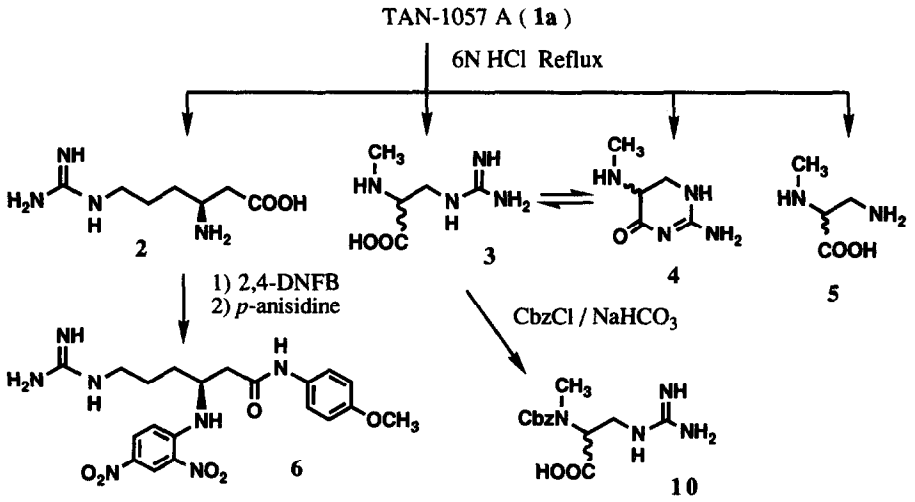


Fig. 3 Acidic Hydrolysis of TAN-1057 A

A new amino acid **3** was synthesized to confirm the structure assumed by spectral and analytical methods, especially by ^{13}C NMR spectroscopy, as shown in Table 2. After the 3-amino group of (2*S*)-N^2-carbobenzoxy(Cbz)-2,3-diaminopropionic acid[6] was protected with the phthaloyl group, the 2-amino group was methylated with iodomethane to give **7**. After deprotection of the phthaloyl group, (2*S*)-N^2-Cbz-N^2-methyl-2,3-diaminopropionic acid **8** was converted into (2*S*)-N^2-Cbz-N^2-methyl-2-amino-3-guanidinopropionic acid **9** by guanidination using *S*-methylisothiourea as shown in Fig. 4. The physicochemical data of **9** agreed with those of **10** (N^2-Cbz derivative of **3**) except for the specific rotation. Thus, **3** as well as **4** and **5** were concluded to be racemic mixtures. The dehydrate form of **3** between the carboxylic acid and the guanidino group is assumed to be suitable for **4** from the SIMS data at *m/z* 143(M+H) and the UV absorption maximum at 232 nm (ε 10,000). The stereochemistry of the 3-position of **2** was determined by the CD method.[7] Amino acid **2** was converted to *N*-dinitrophenyl-β-homoarginine *p*-methoxyanilide **6**, which showed a positive Cotton effect at 402 nm. This indicated the absolute configuration of the 3-position of **2** to be "*S*."

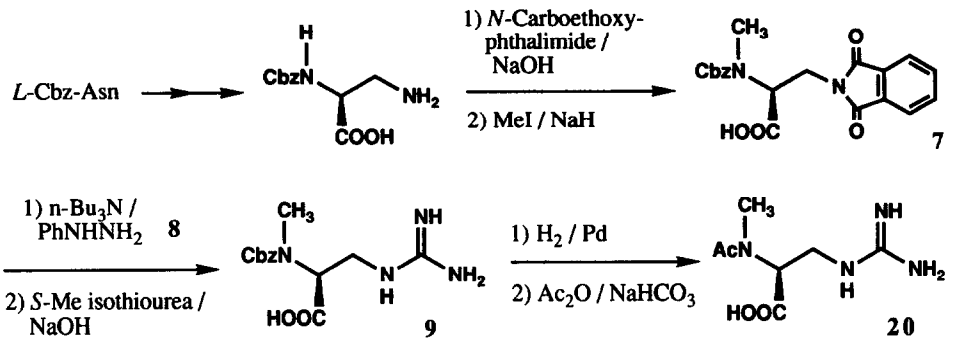


Fig. 4 Synthetic Route of 2,3-Diaminopropionic Acid Analogs

The data proved the presence of free amino and guanidino groups in **1a**. The antibiotic afforded *N*-acetyl derivative **11** and *tert*-butyl carbamate **12** by the Schotten-Baumann reactions. The positive Sakaguchi reaction and carbon signals at δ 157~160 ppm indicated that it had guanidino groups. Treatment of **1a** with acetylacetone in sodium carbonate solution[8] gave an N^6-dimethylpyrimidyl-3-enamino derivative **13**, which was hydrolyzed in 2 N HCl with refluxing for 15 hours to give amino acid **14**. Spectroscopic analysis proved **14** to be N^{ε}-dimethylpyrimidyl-β-lysine as shown in Fig. 5. Thus, the free guanidino and amino groups were attributed to an *L*-β-homoarginine moiety.

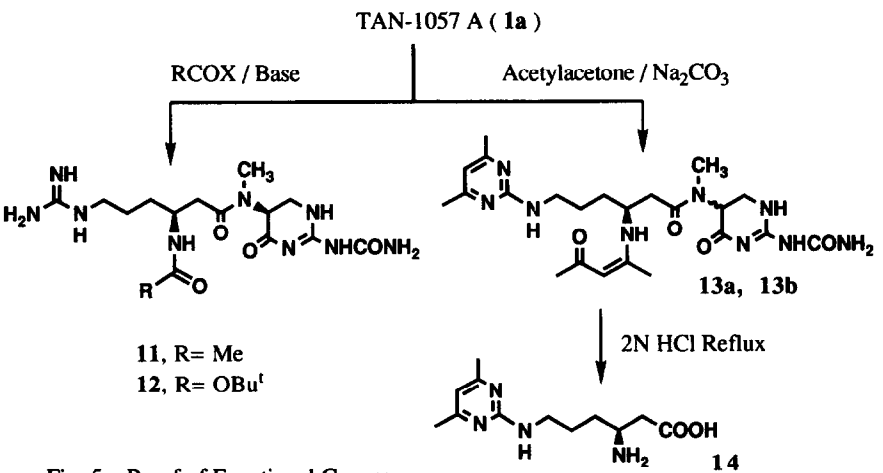


Fig. 5 Proof of Functional Groups

Based on 2D-NMR studies such as H-H COSY, C-H COSY and COLOC, the signals of **1a** were assigned as shown in Fig. 6. The carbonyl carbon signal (δ 175.31 ppm) of the β-homoarginine residue was correlated with the *N*-methyl proton signal (δ 3.07 ppm) of the 2,3-diaminopropionic acid residue. This was evidence of an amide bond between the two residues.

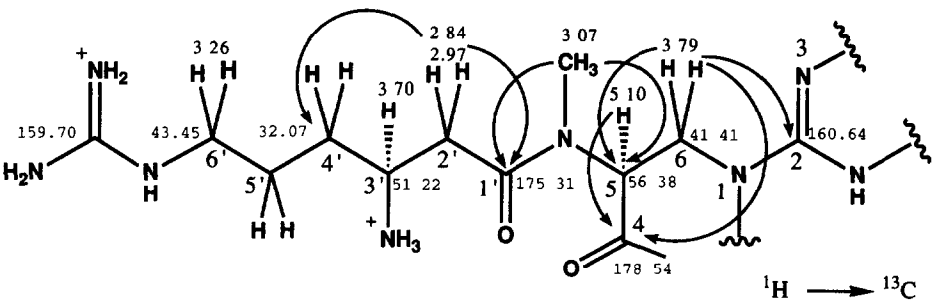
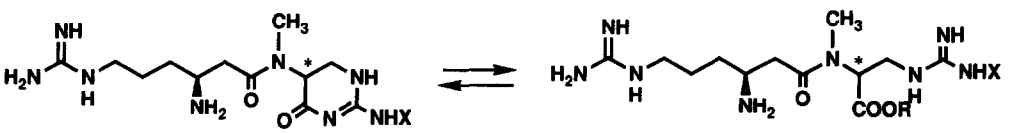


Fig. 6 Long-range C-H Correlation of TAN-1057 A (**1a**)

TAN-1057 A and B gradually lost their antibacterial activities in basic aqueous solutions. Hydrolysis of **1a** in 2% sodium carbonate solution at 70℃ for 1 hour afforded **15**. The molecular formula of **15** was determined to be $C_{13}H_{27}N_9O_4$. No ring structure was confirmed by UV absorption. Compound **15** was a 1:1 epimeric mixture of **15a** and **15b** according to HPLC analysis and the Cotton effect in CD spectra. Conversion of **15** to a methyl ester **16** by HCl / MeOH indicated that **15** had a carboxyl group which had appeared to be due to hydrolysis. The methyl ester **16** could be returned to a mixture of **1a** and **1b** in basic solutions such as triethylamine in methanol. This seemed to result from intramolecular recyclization as shown in Fig. 7.



I (cyclic form) II (hydrolyzed form)

Fig. 7 Hydrolysis and Cyclization of TAN-1057

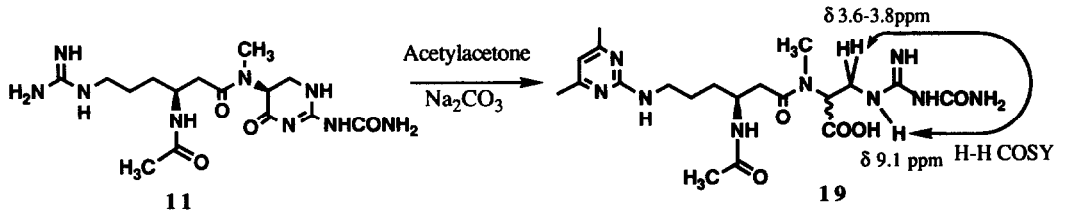
Hydrolysis (I→II)

Substrate (I)	X	a:b Ratio	Conditions	Product (II)	X	R	a:b Ratio
1	$CONH_2$	94:6	2% Na_2CO_3, 70℃	**15**	$CONH_2$	H	1:1
1	$CONH_2$	94:6	0.1 N HCl, 70℃	**15**	$CONH_2$	H	89:11
1	$CONH_2$	94:6	0.1 N HCl, Reflux	**17**	H	H	ca. 2:1

Cyclization (I←II)

Product (I)	X	a:b Ratio	Conditions	Substrate (II)	X	R	a:b Ratio
1	$CONH_2$	1:1	1) 2N HCl / MeOH 2) NaOMe / MeOH	**15**	$CONH_2$	H	1:1
18	H	1:1		**17**	H	H	ca. 2:1

Hydrolysis of **1a** in 0.1~0.2 N HCl with refluxing for 4 hours gave another hydrolysis product **17**, which had the molecular formula of $C_{12}H_{26}N_8O_3$. Hydrolysis and decarbamoylation seemed to produce **17**. Compound **17** was converted *via* the corresponding methyl ester to a cyclization product **18**, which showed UV absorption at 232 nm (ε 6,100) similar to that of **4**. These findings indicated that the eliminated carbamoyl group had been substituted at either the nitrogen atom of the 2-amino group or N^1-atom of 5,6-dihydro-4(1H)-pyrimidone ring. 2D-NMR analyses clarified the nitrogen atom. While H-H COSY of **1a** in DMSO-d_6 showed no cross peak with any of the NH-protons, that of the hydrolyzed form derivative **19** in DMSO-d_6 / TFA showed a cross peak between an NH-proton (δ 9.1 ppm) and the C6-methylene protons (δ 3.6~3.8 ppm), as shown in Fig. 8. These data showed that the carbamoyl group had been substituted onto the 2-amino group. The structures of **1a** and **1b** were thus determined, except for the stereochemistry of the C5 position.

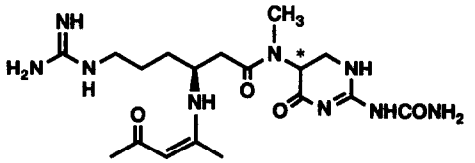


11 **19**

Fig. 8 Position of Carbamoyl Group

Epimerization during the acidic degradation made it impossible to determine the absolute configuration of the C5 position from the degradation products. Optically pure **9**, synthesized from *L*-Asn, was lead to the *N*-acetyl derivative **20**, which showed the negative Cotton effect at 214 nm (θ -30,300) in the CD spectrum. One isomer of hydrolysis product **15a** (de. 78%) showed a similar curve in the CD spectrum. Because a 1:1 mixture of **15a** and **15b** did not show any Cotton effects, the C3' asymmetric center was regarded as having little effect on the CD spectrum. This indicated that **15a** had the same "*S*" configuration as **20** at the C5 position. Since **15a** was obtained with retention of the stereochemistry from **1a**, the absolute structures of **1a** and the epimer **1b** were determined as shown in Fig. 1.

The minor component **1b** was hardly obtained because of the equilibrium. In order to obtain enough **1b** to examine its optical, physicochemical and biological properties, protection followed by separation and deprotection were tried. *N*-Boc and *N*-Cbz derivatives, which were easily deprotected, did not afford separation of the two isomers. On the other hand, the enamino derivative **13** was separable by prep. HPLC and easily deprotected under mild acidic conditions. Thus, the enamino group was selected as a protecting group. Compound **1** (**a**:**b** = 1:1) was treated with acetylacetone and sodium carbonate in methanol at room temperature for 24 hours, giving a mixture of **21a** and **21b** (Fig. 9). Prep. HPLC (mobile phase: 7% acetonitrile / 0.01 M phosphate buffer; pH 6.3, column: ODS; YMC-pack S-363) separated the mixture into two fractions. Each one was passed through a column of IRA-402 (Cl), kept at pH 3.0 at 4℃ for 2 hours to deprotect the enamino group and desalinated at pH 7.0 to give **1a** and **1b**, respectively.



*S, **21a**
*R, **21b**

Fig. 9 Enamino-derivatives of TAN-1057

Another TAN-1057 producing organism, *Flexibacter* sp. PK-176[2], was discovered to produce two minor active components. They were thought to be related to **1** and were named TAN-1057 C and D (**22b**, Rt = 11.5 min and **22a**, Rt = 16.7 min; HPLC conditions, as listed in Table 2, mobile phase 1). They had the same molecular formulas and C-C bond sequences as **1** from the physicochemical data, but were not as stable as **1** in aqueous solutions. In comparison of the ^{13}C NMR spectra of **1a** and **22b**, differences in chemical shifts were conspicuous at the C2', C5 and C6 carbons (Table 2). From the NMR analysis, **1a** had three protons substituted on the $N^{3'}$-atom appearing at δ 8.10 ppm as a broad singlet, while **22b** had only one appearing at δ 8.08 ppm as a doublet (J = 4 Hz). This indicated that the 3'-amino group formed amide bond in **22b**. From these findings, **22b** was deduced to have a 7-membered amide ring as shown in Fig. 10. The amide carbonyl and the endo-guanidino =NH group were located at the 1-6 position, making it easy for reannulation to a 6-membered ring to occur in basic solutions. Compound **22b** changed into **1b** and **1a** in basic solutions and **1b** was detected as the major product early in the reaction. Thus, the stereochemistry at the C5 position of **22b** was concluded to be "*R*". From comparison of the CD spectra of **22b** and **22a**, this was elucidated to be the epimer at the C5 position.

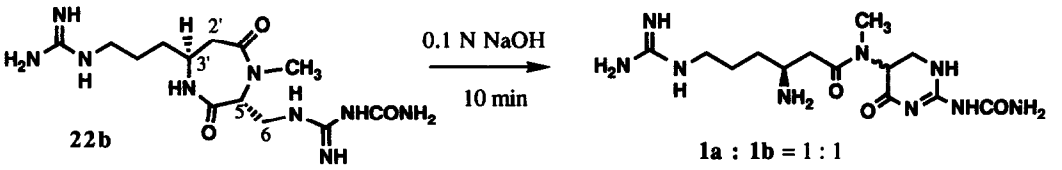


Fig. 10 Conversion of TAN-1057 C to TAN-1057 B and A

TAN-1057 showed broad antibacterial activities against Gram-positive bacteria, including MRSA. They showed weak inhibitory activities against Gram-negative bacteria. In an efficacy study with *Staphylococcus aureus*-infected mice, **1a** showed remarkable therapeutic effects when administered subcutaneously and orally. The ED_{50} values were lower than those of vancomycin (VCM) and imipenem/cilastatin (IPM / CS), even in the MRSA-infected model (Table 3). The preliminary acute toxicities (LD_{50}) of **1a** in mice were ca. 50 mg/kg for intraperitoneal administration or intravenous injection, ca. 100 mg/kg for subcutaneous administration and >400 mg/kg for oral administration.

Table 3 Antibacterial Activities of TAN-1057 A in Mice[2)]

Microorganism	Inoculum (CFU / mouse)	Antibiotic	MIC[a)] (μg/ml)	ED_{50}[c)] (mg/kg) *sc*	*po*
S. aureus 308A-1	10^8	TAN-1057 A	12.5	0.027	1.20
		IPM / CS	0.025	0.10	
		VCM	0.78	2.2	
S. aureus N133A (MRSA)	10^8	TAN-1057 A	6.25	0.026	0.56
		IPM / CS	>25	4.2	
		VCM	1.56	2.3	
S. aureus N295A (MRSA)	10^8	TAN-1057 A	12.5	0.064	1.56
Streptococcus pneumoniae type1	10^1	TAN-1057 A	6.25[b)]	>25	

a) MIC values were determined by an broth dilution method using Mueller-Hinton broth medium, inoculum size, 10^6 CFU/ml.

b) MIC value was determined by an agar dilution method using Trypticase soy agar medium; inoculum size, 10^8 CFU/ml

c) Mice were infected intraperitoneally with 0 5 ml of bacterial suspension. Groups of five mice at each dosage level were given 0 2 ml of antibiotics solution immediately after infection The ED_{50} was calculated from the survival rate 5 days after infection.

Experimental

The specific optical rotations, UV and CD (JASCO J-20A with DP-501N) spectra were measured at 23~28℃ in water unless otherwise stated. The IR spectra were measured in KBr pellets and reported in the carbonyl region. The δ values in the ^{1}H NMR (300 MHz) and ^{13}C NMR (75 MHz) were recorded in ppm downfield from sodium 3-trimethylsilylpropionate-d_4 or TMS using a Bruker AC-300 spectrometer. The multiplicities were abbreviated as follows: s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, Q = quaternary carbon. The SIMS were measured with a Hitachi M-80A mass spectrometer with a xenon ion beam source. The samples were dissolved or suspended in glycerol.

Isolation of 1a and 1b: The culture broth of *Flexibacter* sp. PK-74 was filtered through Hyflo Super Cel^R. The filtrate (85 l) was loaded onto a column of Amberlite^R IRC-50 (Na^+ type, 2.5 l) and the active substances were eluted with 0.2 N HCl (25 l). The eluate was subjected to carbon chromatography (1.5 l) eluting with 8% *iso*-BuOH (7.5 l). The eluate was chromatographed on CM-Sephadex^R C-25 (Na^+ type, 250 ml) eluting with 0.2 M NaCl aq. (4.5 l). The active fraction was desalted with carbon (0.2 l) and lyophilized to give a crude powder of **1** (4.1 g). The crude powder obtained in the same manner (6.0 g) was subjected to prep. HPLC (mobile phase: 0.01 M phosphate buffer; pH 6.3, column: ODS; YMC-pack^R R-355). The active fraction was passed through Amberlite^R IRA-402 (Cl^- type, 100 ml) and desalted with carbon (150 ml) to give a 2 HCl salt of **1a** (1.56 g) as a white powder. The **1b**-containing fraction, obtained by the prep. HPLC, was subjected to prep. HPLC (mobile phase: 0.1 M phosphate buffer; pH 5.0, column: YMC-pack D-ODS-5) three times. The pure fraction was treated in the same manner to afford a 2 HCl salt of **1b** (5 mg) as a white powder. **1a** (2HCl); ^{1}H NMR (D_2O): 1.76 (4H, m), 2.84 (1H, dd, J =18, 8.5Hz), 2.99 (1H, dd, J = 18, 4Hz), 3.14 (3H, s), 3.25 (2H, t, J = 6Hz), 3.68 (1H, m), 3.93 (2H, m), 5.11 (1H, dd, J = 12.5, 9Hz).

Epimerization of 1a: A solution of **1a** (0.50 g, purity 89%) in MeOH (20 ml) was stirred with 28% MeONa / MeOH (1.06 ml) at r.t. for 20 hr. The reaction mixture was neutralized and desalted with carbon (26 ml) to give a mixture of **1a** and **1b** (1:1, 335 mg, y. 75%) as a white powder, $[\alpha]_D^{28}$ +15.1° (c 0.478). UV: λmax 215 nm ($E^{1\%}$ 512), 245 (258). ^{1}H NMR (D_2O); 1.76 (4H, m), 2.80 (1H, m), 2.98 (1H, m), 3.07/3.08 (3H, s), 3.26 (2H, t, J = 6Hz), 3.68 (1H, m), 3.73 (1H, m), 3.80 (1H, m), 5.08 (1H, m). Anal. Calcd. for $C_{13}H_{25}N_9O_3 \cdot 2HCl \cdot 2.5H_2O$: C, 32.99; H, 6.81; N, 26.63. Found: C, 33.18; H, 7.11; N, 26.56.

Acidic degradation of 1a: **1a** (1.0 g) was refluxed in 6 N HCl (50 ml) for 18 hr. The reaction mixture was evaporated and chromatographed on Dowex^R 50WX2 (50~100 mesh, H^+ type, 40 ml) eluted with stepwise gradient of 0.4, 0.8, 1.0, 1.5 and 2.0 N HCl. The pure fractions containing **2** and **5** were evaporated to give **2** (474 mg) and **5** (51 mg), respectively. Mixtures of **2~4** (534 mg) and **3~5** (170 mg) were independently rechromatographed in the same manner to give **2** (160 mg), **5** (27 mg) and a mixture of **3** and **4** (429 mg). The powder of **5** was crystallized from MeOH / Et_2O / hexane to afford **5** (66 mg) as colorless crystals. The mixture was crystallized from MeOH / Et_2O to afford **4** (39 mg) as colorless crystals. The mother liquor was triturated from Et_2O to give **3** (320 mg). **2**: $[\alpha]_D^{28}$ +13.4° (c 0.491). SIMS: *m/z* 189 (M+H). ^{1}H NMR (D_2O); 1.73 (4H, m), 2.66 (1H, dd, J = 17.4, 7.8Hz), 2.79 (1H, dd, J = 17.4, 4.8Hz), 3.23 (2H, t, J = 6.4Hz), 3.63 (1H, m). Anal. Calcd. for $C_7H_{16}N_4O_2 \cdot 2HCl \cdot H_2O$: C, 30.12; H, 7.22; N, 20.07; Cl, 25.40.

Found: C, 30.40; H, 7.38; N, 19.80; Cl, 26.05. **3**: $[\alpha]_D^{24}$ +2.3°(c 0.51). SIMS: *m/z* 161 (M+H), UV: end. ^{1}H NMR (D_2O); 2.82 (3H, s), 3.84 (1H, dd, J = 15.0, 5.1Hz), 3.90 (1H, dd, J = 15.0, 5.1Hz), 4.10 (1H, t, J = 5.1Hz). Anal. Calcd. for $C_5H_{12}N_4O_2 \cdot 2HCl \cdot 0.5H_2O$: C, 24.81; H, 6.24; N, 23.14. Found: C, 25.05; H, 6.10; N, 23.00. **4**: $[\alpha]_D^{24}$ -3.1°(c 0.32). SIMS: *m/z* 143 (M+H), UV: λmax 232 nm ($E^{1\%}$ 465). ^{1}H NMR (D_2O); 2.88 (3H, s), 3.77 (1H, t, J = 12.8Hz), 4.15 (1H, dd, J = 12.8, 7.0Hz), 4.59 (1H, dd, J = 12.8, 7.0Hz). Anal. Calcd. for $C_5H_{10}N_4O \cdot 2HCl$: C, 27.92; H, 5.62; N,26.05. Found: C, 27.50; H, 5.55; N, 25.75. **5**: $[\alpha]_D^{24}$ 0.0° (c 0.48). SIMS: *m/z* 119 (M+H), UV: end. ^{1}H NMR (D_2O); 2.84 (3H, s), 3.52 (1H, dd, J = 13.3, 9.1Hz), 3.60 (1H, dd, J = 13.3, 4.8Hz), 4.13 (1H, dd, J = 9.1, 4.8Hz). Ref.[4]: δ ppm downfield from DSS in D_2O; 2.93 (3H, s), 3.60 (2H, d), 4.21 (1H, t) as monohydrochloride. Anal. Calcd. for $C_4H_{10}N_2O_2 \cdot 2HCl$: C, 25.15; H, 6.33; N, 14.66. Found: C, 25.06; H, 6.38; N, 14.67.

***N*-2,4-Dinitrophenyl-β-homoarginine *p*-methoxyanilide (6)**: A solution of **2** (140 mg) in 2% $NaHCO_3$ (5 ml) was adjusted to pH 9.0, mixed with EtOH (3 ml) and 2,4-dinitrofluorobenzene (2,4-DNFB, 0.093 ml) and stirred at r.t. for 15 hr. The reaction mixture was diluted with water and washed with Et_2O (15 ml, twice). The aqueous layer was neutralized and evaporated to give N^{β}-2,4-dinitrophenyl-β-homoarginine (167 mg, y. 94%). This (126 mg) was dissolved in DMF (2 ml) and stirred with *p*-anisidine hydrochloride (62.4 mg), Et_3N (0.075 ml), 1-hydroxybenzotriazole (58 mg) and dicyclohexylcarbodiimide (88 mg) at 0℃ for 30 min and then at r.t. for 30 min. The reaction mixture was filtered and the filtrate was evaporated. The residue was subjected to CM-Sephadex C-25 (Na^+ type, 30 ml) column chromatography eluted with 0.2 M NaCl aq. and 1.0 N HCl. The eluate was adjusted to pH 6.5 and evaporated to give **6** (76 mg, 43%). CD: [θ] 261nm -14100, [θ] 340 -3200, [θ] 402 +5500 / MeOH, UV: λmax 251 nm ($E^{1\%}$ 524), 346 (403), 416 (121) / MeOH. ^{1}H NMR (DMSO-d_6); 1.57 (2H, m), 1.73 (2H, m), 2.74 (2H, m), 3.11 (2H, m), 3.71 (3H, s), 4.38 (1H, m), 6.5~7.5 (4H, br), 6.86 (2H, d, J = 9.0Hz), 7.39 (1H, d, J = 9.9Hz), 7.46 (2H, d, J = 9.0Hz), 7.61 (1H, br), 8.26 (1H, dd, J = 9.9, 2.6Hz), 8.86 (1H, d, J = 2.6Hz), 9.09 (1H, d, J = 8.6Hz), 10.05 (1H, br). Anal. Calcd. for $C_{20}H_{25}N_7O_6 \cdot HCl$: C, 48.44; H, 5.28; N, 19.77; Cl, 7.15. Found: C, 48.09; H, 5.19; N, 19.40; Cl, 7.04.

(2*S*)-N^2-Cbz-N^3-Phthaloyl-2,3-diaminopropionic acid: (2*S*)-N^2-Cbz-2,3-diaminopropionic acid (4.67 g) was stirred with *N*-carboethoxyphthalimide (4.5 g) in 1.8% Na_2CO_3 aq.(120 ml) at r.t. for 2 hr. The reaction mixture was washed with Et_2O (50 ml, three times), adjusted to pH 2.3 and extracted with EtOAc (100 ml). The extract was triturated from Et_2O / petro. benzin (*d* = 0.671) to give (2*S*)-N^2-Cbz-N^3-phthaloyl-2,3-diaminopropionic acid (4.48 g, y. 61%). ^{1}H NMR (DMSO-d_6); 3.89 (1H, dd, J = 14.0, 8.3Hz), 3.97 (1H, dd, J = 14.0, 6.3Hz), 4.34 (1H, m), 4.97 (1H, d, J = 12.7 Hz), 4.99 (1H, d, J = 12.5 Hz), 7.20~7.40 (5H, m), 7.87 (1H, d, J = 3.3Hz), 7.8~7.95 (4H, m). Anal. Calcd. for $C_{19}H_{16}N_2O_6$: C, 61.96; H, 4.38; N, 7.61. Found: C, 61.78; H,4.36; N,7.50.

(2*S*)-N^2-Cbz-N^2-Methyl-N^3-phthaloyl-2,3-diaminopropionic acid (7): A solution of (2*S*)-N^2-Cbz-N^3-phthaloyl-2,3-diaminopropionic acid (3.08 g) in THF (37 ml) was mixed at 0℃ with MeI (4.2 ml) and NaH (60% oil suspension, 1.05 g). The mixture was stirred at r.t. for 2 days. The reaction mixture was poured into 0.1 M phosphate buffer (pH 3.0, 300 ml) and extracted in the manner described above. The extract

was washed with 5% sodium thiosulfate (three times) and water (three times), dried over Na_2SO_4 and evaporated to give a crude powder (2.35 g). The powder was purified with Sephadex^R LH-20 (1.0 l, MeOH) to give **7** (1.76 g, y. 55%). ^{1}H NMR (DMSO-d_6); 2.88/2.91 (3H, s, s-*trans*/s-*cis* at N-CO bond), 3.9~4.2 (2H, m), 4.75~5.05 (3H, m), 7.05~7.27 (5H, m), 7.80~7.95 (4H, m). Anal. Calcd. for $C_{20}H_{18}N_2O_6$: C, 62.82; H, 4.74; N, 7.33. Found: C, 62.13; H, 4.84; N, 7.13.

(2*S*)-N^2-Cbz-N^2-Methyl-2,3-diaminopropionic acid (8): A solution of **7** (1.7 g) in EtOH (5 ml) was mixed with *n*-Bu_3N (1.06 ml) and $PhNHNH_2$ (2.19 ml) and then refluxed for 6 hr. The reaction mixture was mixed with methyl ethyl ketone (10 ml) and refluxed for 15 min. The mixture was cooled to r.t., mixed with AcOH (0.38 ml) and kept at 4℃. The precipitate was collected, suspended in MeOH and filtered. The filtrate was concentrated and triturated from methyl ethyl ketone to give **8** (518 mg, y. 46%). ^{1}H NMR (DMSO-d_6 / TFA); 2.89/2.90 (3H, s, s-*trans*/s-*cis* at N-CO bond), 3.32 (2H, m), 4.64 (1H, m), 5.00~5.20 (2H, m), 7.25~7.50 (5H, m), 7.92 (3H, br). Anal. Calcd. for $C_{12}H_{16}N_2O_4$: C, 57.13; H, 6.39; N, 11.10. Found: C, 56.82; H, 6.35; N, 10.82.

(2*S*)-N^2-Cbz-N^2-Methyl-2-amino-3-guanidinopropionic acid (9): A suspension of **8** (390 mg) in water (3.9 ml) was mixed with *S*-methylisothiourea sulfate (1.1 g), then adjusted to pH 10.9 with 2 N NaOH and stirred at r.t. for 8 hr. Additional *S*-methylisothiourea sulfate (220 mg) was added and stirring was continued at pH 10.9 for 14 hr. The reaction mixture was neutralized and chromatographed on Diaion^R HP-20 (50~100 mesh, 50 ml) to give **9** (308 mg, y. 68%), $[\alpha]_D^{26}$ -48.5° (c 0.400, 50% MeOH aq.). ^{1}H NMR (DMSO-d_6/TFA); 2.82/2.86 (3H, s, s-*trans*/s-*cis* at N-CO bond), 3.40~3.75 (2H, m), 4.67 (1H, m), 5.00~5.20 (2H, m), 7.25~7.45 (5H, m), 7.47 (1H, br). Anal. Calcd. for $C_{13}H_{18}N_4O_4$: C, 53.05; H, 6.16; N, 19.04. Found: C, 52.72; H, 6.08; N, 18.88.

Benzyloxycarbonylation of 3 to 10: A solution of **3** (64 mg) in 2% $NaHCO_3$ aq.(5 ml) was stirred with CbzCl (0.04 ml) at r.t. for 1.5 hr. $NaHCO_3$ (50 mg) and CbzCl (0.02 ml) were added and the mixture was stirred for 3 hr. The reaction mixture was washed with Et_2O (twice), adjusted to pH 7 and concentrated. Precipitates were collected and purified with Diaion HP-20 (50~100 mesh, 10 ml) to afford **10** (44 mg, y. 54%), $[\alpha]_D^{24}$ -3.7° (c 0.43, 50% MeOH aq.). Anal. Calcd. for $C_{13}H_{18}N_4O_4$: C, 53.05; H, 6.16; N, 19.04. Found: C, 52.79; H, 6.15; N, 18.91

Acetylation of 1a to 11: A crude powder of **1a** (1.0 g, purity ca.60%) was stirred with acetic anhydride (11.8 ml) in 2% $NaHCO_3$ aq.(100 ml) at r.t. for 16 hr. The reaction mixture was passed through IRA-402 (Cl^- type, 50 ml), and chromatographed on carbon (20 ml), giving **11** (700 mg, y. quant.) as a white powder, $[\alpha]_D^{27}$ -42.9° (c 0.555). SIMS: *m/z* 398 (M+H), 436 (M+K), CD: [θ]269nm -10350, [θ]231 -13170, [θ]213 +11290, UV: λmax 213 nm ($E^{1\%}$ 445), 245 (210). IR: 1720, 1660, 1540 cm^{-1}, ^{1}H NMR(D_2O): 1.59 (2H, m), 1.64 (2H, m), 1.99 (3H, s), 2.69 (2H, d, J = 7Hz), 3.10 (3H, s), 3.20 (2H, t, J = 5Hz), 3.68 (1H, dd, J = 13, 9Hz), 3.75 (1H, t, J = 13Hz), 4.19 (1H, m), 5.01 (1H, dd,J = 13, 9Hz). ^{13}C NMR (D_2O: 178.28 (Q), 176.51 (Q), 176.40 (Q), 162.45 (Q), 160.58 (Q), 159.64 (Q), 56.56 (CH), 49.20 (CH), 43.54 (CH_2), 41.37 (CH_2), 41.15 (CH_2), 37.50 (CH_3), 33.77 (CH_2), 27.39 (CH_2), 24.85 (CH_3). Anal. Calcd. for

$C_{15}H_{27}N_9O_4 \cdot 1.5HCl \cdot H_2O$: C, 38.32; H, 6.32; N, 26.81; Cl, 11.31. Found: C, 38.05; H, 6.47; N, 26.66; Cl, 11.20.

***tert*-Butoxycarbonylation of 1a to 12**: A crude powder of **1a** (2.0 g, purity 89%) was stirred with Et_3N (1.07 ml) and 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitorile (1.04 g) in 50% dioxane aq. (100 ml) at r.t. for 20 hr. The reaction mixture was concentrated and diluted with water (50 ml). The aqueous layer was adjusted to pH 6.0, washed with EtOAc (100 ml, twice) and chromatographed on carbon (26 ml) to give a crude powder of **12** (833 mg). This powder was purified by prep. HPLC (ODS, YMC-pack S-363). The pure fraction was passed through IRA-402 (Cl^- type, 200 ml) and desalted with Diaion^R SP-207 (20 ml) to give **12** (222 mg, y.11%) as a white powder. SIMS: *m/z* 456 (M+H). IR: 1720, 1670, 1610 cm^{-1}. Anal. Calcd. for $C_{18}H_{33}N_9O_5 \cdot 2HCl$: C, 40.91; H, 6.68; N, 23.86. Found: C, 40.73; H, 7.21; N, 23.47.

Pyrimidylation of 1a to 13: A crude powder of **1a** (4.19 g, purity 89%) was stirred with acetylacetone (3.6 ml) and K_2CO_3 (4.1 g) in water (60 ml) at r.t. for 24 hr. Precipitates were collected, washed with water and dried in vacuo to give **13** (**a**:**b** = 1:1, 2.26 g, y. 49%) as a white powder. SIMS: *m/z* 502 (M+H), 540 (M+K). UV: λmax 214 nm ($E^{1\%}$ 491), 238 (546), 309 (462) / MeOH, IR: 1720, 1580 cm^{-1}, ^1H NMR (DMSO-d_6); 1.61 (4H, m), 1.87 (3H, s), 1.99/2.00 (3H, s), 2.40 (6H, s), 2.67 (1H, m), 2.72 (1H, m), 2.98/3.00 (3H, s), 3.43 (2H, brs), 3.68 (1H, m), 3.78 (1H, m), 4.90 (1H, s), 5.22 (1/2H, dd, J = 12, 8Hz), 5.36 (1/2H, dd, J = 13, 8Hz), 6.75 (1H, s), 7.2 (br), 7.6 (br), 8.94 (1H, brs), 10.69 (1H, brd). Anal. Calcd. for $C_{23}H_{35}N_9O_4 \cdot 3H_2O$: C, 49.72; H, 7.44; N, 22.69. Found: C, 49.56; H, 6.94; N, 22.68.

Acidic hydrolysis of 13 to 14: A solution of **13** (**a**:**b** = 1:1, 115 mg) in 2 N HCl (10 ml) was refluxed for 15 hr. The reaction mixture was neutralized and chromatographed on carbon (10 ml) to give **14** (19 mg, y. 37%). UV: λmax 236 nm ($E^{1\%}$ 502), 300 (156). ^1H NMR (D_2O); 1.74 (4H, m), 2.34 (6H, s), 2.47 (1H, dd, J = 17, 8Hz), 2.59 (1H, dd, J = 17, 5Hz), 3.43 (2H, t, J = 5Hz), 3.55 (1H, m), 6.57 (1H, s). Anal. Calcd. for $C_{12}H_{20}N_4O_2 \cdot 2H_2O$: C, 49.99; H, 8.39; N, 19.43. Found: C, 50.05; H, 8.23; N, 19.82.

Basic hydrolysis of 1a to 15: A crude powder of **1a** (20.07 g, purity 60%) was dissolved in 2% Na_2CO_3 (600 ml) and heated at 70℃ for 2 hr. The reaction mixture was desalted with carbon (600 ml) to afford **15** (**a**:**b** = 1:1, 13.63 g) as a white powder.

Acidic hydrolysis of 1a to 15a: A sulfate of **1** (1.10 g, purity 82%, **a**:**b** = 94: 6) was stirred in 0.1 N HCl (60 ml) at r.t. for 16 hr and heated at 70℃ for 4 hr. The reaction mixture was adjusted to pH 7.0 and chromatographed on Amberlite^R CG-50 (Na^+ type, 15 ml) eluted with NaCl aq. gradient. The pure fraction was desalinated with carbon (30 ml) to give **15** (**a**:**b** = 89:11, 376 mg, y. 42%) as a white powder, $[\alpha]_D^{23}$ -15.7° (c 0.426). SIMS: *m/z* 374 (M+H), CD: [θ]215nm -20900. IR: 1700~1600 cm^{-1}, ^1H NMR (D_2O); 1.78 (4H, m), 2.85 (1H, dd, J = 18, 7Hz), 2.96 (1H, dd, J = 18, 5Hz), 3.01 (3H, s), 3.25 (2H, t, J = 6Hz), 3.66 (1H, dd, J = 14, 8.5Hz), 3.69 (1H, m), 3.85 (1H, dd, J = 14, 6.5Hz), 5.01 (1H, m). Anal. Calcd. for $C_{13}H_{27}N_9O_4 \cdot 2HCl \cdot 1.5H_2O$: C, 32.99; H, 6.81; N, 26.63; Cl, 14.98. Found: C, 33.29; H, 6.75; N, 26.50; Cl, 15.22.

Methyl esterification of 15 to 16: 15 (**a**:**b** = 1:1, 492 mg) was dissolved in 2 N HCl / MeOH (16.5 ml) and stirred at r.t. for 17 hr. The reaction mixture was concentrated, the azeotrope was formed with MeOH (10 ml, three times) and water (twice), and then this was freeze-dried to obtain a powder. The powder was washed with acetone to give **16** (**a**:**b** = 1:1, 613 mg, y. quant.) as a white powder. SIMS: *m/z* 388 (M+H). IR: 1730, 1670, 1640 cm^{-1}, ^1H NMR (D_2O); 1.74 (4H, m), 2.84 (1H, dd, J = 18, 8.5 Hz), 2.99 (1H, dd, J = 18, 4Hz), 3.11 (3H, s), 3.25 (2H, t, J = 6.5Hz), 3.67 (1H, m), 3.79 (3H, s), 3.80 (1H, m), 3.96 (1H, dd, J = 15, 5.5Hz), 4.94 (1H, dd, J = 8.5, 5.5Hz). Anal. Calcd. for $C_{14}H_{29}N_9O_4 \cdot 3HCl \cdot 1.5H_2O$: C, 32.10; H, 6.73; N, 24.06; Cl, 20.30. Found: C, 32.03; H, 6.95; N, 23.73; Cl, 20.94.

Intramolecular cyclization of 16: 16 (**a**:**b** = 1:1, 247 mg) was stirred in MeOH (8 ml) with 28% NaOMe / MeOH (0.45 ml) at r.t. for 24 hr. The reaction mixture was diluted with water (20 ml), immediately adjusted to pH 7.0 and desalted with carbon (18 ml) to give a 1 : 1 mixture of **1a** and **1b** (173 mg, y. 75%) as a white powder. UV: λmax 214 nm ($E^{1\%}$ 420), 245 (200). ^1H NMR (D_2O); 1.76 (4H, m), 2.80 (1H, m), 2.99 (1H,m), 3.08/3.09 (3H, s), 3.26 (2H, t, J = 6Hz), 3.70 (1H, m), 3.72 (1H, m), 3.81 (1H, m), 5.09 (1H, m).

Decarbamoylation of 1a to 17: 1a (4.1 g, purity 66%) was dissolved in 0.2 N HCl (149 ml) and heated at 100℃ for 40 hr. The reaction mixture was worked up by the procedure described for the acidic hydrolysis of **1a** to give **17** (**a**:**b** = ca. 2:1, 1.39 g, y.58%) as a white powder. SIMS: *m/z* 331 (M+H), UV: λmax 221 nm ($E^{1\%}$ 80) / 0.1 N NaOH. IR: 1660~1600 cm^{-1}, ^1H NMR (D_2O); 1.77 (4H, m), 2.78 (1H, dd, J = 18.5, 8.5Hz), 2.95 (1H, dd, J = 18.5, 4Hz), 2.99/3.02 (3H, s), 3.26 (2H, t, J = 6.5Hz), 3.55 (1H, dd, J =15, 9Hz), 3.70 (1H, m), 3.74 (1H, dd, J = 15, 5.5Hz), 4.94 (1H, dd, J = 9, 5.5Hz). Anal. Calcd. for $C_{12}H_{26}N_8O_3 \cdot 2.5HCl \cdot 1.5H_2O$, C, 32.13; H, 7.08; N, 24.98; Cl, 19.76. Found: C, 31.97; H, 6.85; N, 25.21; Cl, 20.62.

Methyl esterification of 17: 17 (**a**:**b** = ca. 2:1, 705 mg) was converted to its methyl ester in the same manner in the methyl esterification of **15** to give **17** methyl ester (672 mg, y. 85%) as a white powder. SIMS: *m/z* 345 (M+H). ^1H NMR (D_2O); 1.74 (4H, m), 2.83 (1H, d, J = 18, 8.5Hz), 3.00 (1H, dd, J = 18, 4.5Hz), 3.10 (3H, s), 3.25 (2H, t, J = 6.5Hz), 3.68 (1H, m), 3.69 (1H, dd, J =15, 9Hz), 3.78 (3H, s), 3.85 (1H, dd, J = 15, 5.5Hz), 4.85 (1H, dd, J = 9, 5.5Hz). Anal. Calcd. for $C_{13}H_{28}N_8O_3 \cdot 3HCl \cdot 0.5H_2O$: C, 33.74; H, 6.97; N, 24.21; Cl, 22.98. Found: C, 33.56; H, 7.36; N, 23.86; Cl, 23.65.

Intramolecular cyclization of 17 methyl ester to 18: A solution of **17** methyl ester (392 mg) in MeOH (13 ml) was stirred with Et_3N (0.48 ml) at r.t. for 24 hr. The reaction mixture was evaporated, the azeotrope was formed with water (10 ml, twice), and this was freeze-dried to give a powder. This powder was washed with acetone to obtain **18** (**a**:**b** = 1:1, 407 mg, y. quant.) as a white powder. SIMS: *m/z* 313(M+H), UV: λmax 232 nm ($E^{1\%}$ 145, sh). IR: 1740, 1700, 1640 cm^{-1}, ^1H NMR (D_2O); 1.76 (4H, m), 2.84 (1H, m), 2.99 (1H, m), 3.10 (3H, s), 3.26 (2H, t, J =6Hz), 3.70 (1H, dd, J = 12.5, 3Hz), 3.71 (1H, m), 3.78 (1H, dd, J = 12.5, 6Hz). Anal. Calcd. for $C_{12}H_{24}N_8O_2 \cdot 3HCl \cdot 0.5H_2O$: C, 33.46; H, 6.55; N, 26.01; Cl, 24.69. Found: C, 33.41; H, 6.73; N, 26.30; Cl, 22.71.

Pyrimidylation and hydrolysis of 11 to 19: A solution of **11** (117 mg) in 2% Na_2CO_3 aq. (3.9 ml) was stirred with acetylacetone (0.13 ml) at r.t. for 3 days. The solution was mixed with K_2CO_3 (100 mg) and stirred for 30 hr. The reaction mixture was neutralized and purified by column chromatography on Diaion HP-20 (50~100 mesh, 12 ml) to give **19** (**a**:**b** = 1:1, 45 mg, y. 36%%) as a white powder. SIMS: *m/z* 480 (M+H), UV: λmax 235 nm ($E^{1\%}$ 347), 300 (89). IR: 1730~1560 cm^{-1}, ^1H NMR (DMSO-d_6/TFA); 1.50 4H, m), 1.81 (3H, s), 2.41 (6H, s), 2.55 (2H, brs), 2.97/2.99 (3H, s), 3.39 (2H, brs), 3.53 (1H, m), 3.79 (1H, m), 4.09 (1H, brs), 4.86/5.04 (1H, m), 6.73 (1H, s), 7.79 (1H, quint), 8.69 (2H, brs), 8.95 (1H, brs), 9.07 (1H, brs), 10.68/10.73 (1H, brs). Anal. Calcd. for $C_{20}H_{33}N_9O_5 \cdot H_2O$: C, 48.28; H, 7.09; N, 25.34. Found: C, 48.25; H, 7.12; N, 24.95.

(2*S*)-N^2-Acetyl-N^2-methyl-2-amino-3-guanidinopropionic acid (20): A solution of **9** (68 mg) in 70% MeOH aq.(15 ml) was stirred with Pd-black (20 mg) under a H_2 atmosphere at r.t. for 1 hr. The reaction mixture was filtered and the filtrate was concentrated. The concentrate was stirred in 0.5% $NaHCO_3$ (10 ml) with acetic anhydride (0.04 ml) at r.t. for 2 hr. The reaction mixture was neutralized and chromatographed on Dowex 50WX2 (50~100 mesh, H^+ type, 5 ml) to obtain a powder (43 mg). This powder was purified with Sephadex LH-20 (90 ml) eluted with 50% MeOH aq. to afford **20** (39 mg, y. 83%). CD: [θ]214 nm -30300. ^1H NMR (D_2O); 2.15/2.17 (3H, s, s-*trans*/s-*cis* at N-CO bond), 2.80/2.99 (3H, s, s-*trans*/s-*cis* at N-CO bond), 3.45~3.90 (2H, m), 4.59/4.95 (1H, dd). Anal. Calcd. for $C_7H_{14}N_4O_3 \cdot 0.5H_2O$: C, 39.81; H, 7.16; N, 26.53. Found: C, 40.16; H, 7.37; N, 26.01.

Separation of 1a and 1b *via* modification: A solution of a mixture of **1a** and **1b** (1.12 g) in MeOH (30 ml) was stirred with Na_2CO_3 (1.10 g) and acetylacetone (1.07 ml) at r.t. for 24 hr. The reaction mixture was diluted with water (50 ml) and immediately neutralized. The aqueous solution was desalted with carbon (20 ml) and subjected to prep. HPLC (ODS, YMC-pack S-363). Two fractions (**21a** and **21b**) were independently passed through IRA-402 (Cl^- type), adjusted to pH 3.0 and stirred at r.t. for 3 hr. Both reaction mixtures were adjusted to pH 6.0 and desalinated with carbon (15 ml) to obtain **1a** (210 mg, y. 19%) and **1b** (211 mg, y. 19%) as white powders, respectively. **1a**: Anal. Calcd. for $C_{13}H_{25}N_9O_3 \cdot 2HCl \cdot 1.5H_2O$: C, 34.29; H, 6.64; N, 27.68; Cl, 15.57. Found: C, 34.29; H, 6.78; N, 27.71; Cl, 17.17. **1b**: ^1H NMR (D_2O): 1.76 (4H, m), 2.81 (1H, dd, J = 18, 8.5Hz), 3.00 (1H, dd, J = 18, 4Hz), 3.08 (3H, s), 3.25 (2H, t, J = 7Hz), 3.69 (1H, m), 3.79 (2H, m), 5.05 (1H, dd, J = 12, 9.5Hz). Anal. Calcd. for $C_{13}H_{25}N_9O_3 \cdot 2HCl \cdot 1.5H_2O$: C, 34.29; H, 6.64; N, 27.68; Cl, 15.57. Found: C, 34.23; H, 6.60; N, 27.63; Cl,16.65.

Isolation of 22a and 22b: The culture broth of *Flexibacter* sp. PK-176 was treated as described for that of *Flexibacter* sp. PK-74 to obtain a crude powder. This powder (8.0 g) was chromatographed in a series of CG-50 (Type I, Na^+ type, 150 ml), carbon, CM-Sephadex (Na^+ type, 50 ml) and carbon to obtain a crude **22** (343 mg). This powder (300 mg) was subjected to prep. HPLC (mobile phase: 0.1 M phosphate buffer; pH 5.0, column: YMC-pack D-ODS-5) to give two fractions. Both were treated as described for the isolation of **1a** and **1b** and triturated with acetone to give 2 HCl salts of **22b** (95 mg) and **22a** (23 mg), respectively. **22b**: $[\alpha]_D^{23}$ -39.6° (c 0.500). CD: [θ]232 +13700, SIMS: *m/z* 356 (M+H), UV: end. IR: 1730, 1640, 1470 cm^{-1}. ^1H NMR (DMSO-d_6): 1.53 (4H, m), 2.84 (3H, s), 2.90 (1H, m), 2.94 (1H, dd, J = 14, 9Hz), 3.17 (2H, d, J = 5Hz), 3.43 (1H, m), 3.74 (2H, brs), 4.68 (1H, m), 7.2 (4H, m), 7.28 (2H, brs), 7.84 (1H, brs), 8.08 (1H, d,

J = 4Hz), 8.74 (2H, brs), 9.14 (1H, brs), 10.46 (1H, brs). Anal. Calcd. for $C_{13}H_{25}N_9O_3 \cdot 2HCl \cdot 2.5H_2O$: C, 32.99; H, 6.81; N, 26.63; Cl, 14.98. Found: C, 33.05; H, 6.49; N, 26.17; Cl, 16.75. **22a**: $[\alpha]_D$ N.D. CD: [θ]233 -10500, SIMS: *m/z* 356 (M+H), UV: end. IR: 1730, 1650, 1470 cm^{-1}, Anal. Calcd. for $C_{13}H_{25}N_9O_3 \cdot 2HCl \cdot 3.5H_2O$: C, 31.78; H, 6.97; N, 25.65. Found: C, 31.99; H, 6.40; N, 25.43.

Conversion 22b to 1a and 1b: A solution of **22b** (15 mg) in 0.1 N NaOH aq. (15 ml) was stirred at r.t. for 10 min. The reaction mixture was adjusted to pH 5.0, desalted with Microacylizer^R G1 (Asahi Kasei Corp.), concentrated and freeze-dried to obtain a crude powder (17 mg). This powder was purified by prep. HPLC (mobile phase: 0.1 M phosphate buffer; pH 5.0, column: ODS; YMC-pack AM-324). The active fraction was passed through IRA-402 (Cl^- type, 5 ml), desalted with Microacylizer G1, concentrated and lyophilized to obtain a mixture of **1a** and **1b** (8 mg, y. 53%).

Acknowledgements

We thank Dr. H. Okazaki for his encouragement throughout this work. We are grateful to Dr. S. Chiba for the acute toxicity tests.

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